Oligomerization state in solution of the cell cycle regulators p13^{suc1} from the fission yeast and p9^{cksphy} from the myxomycete *Physarum*, two members of the cks family

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Abstract The cks proteins (for cdc2 kinase subunit) are essential cell cycle regulators. They interact strongly with the mitotic cdc2 kinase, but the mechanism and the biological function of this association still await understanding. The oligomerization state in solution of two members of this ubiquitous protein family, the suc1 gene product from the fission yeast and the newly cloned cksphy gene product from the myxomycete *Physarum*, was investigated by small-angle X-ray scattering (SAXS) and biochemical methods. We found that the major molecular species are monodispersed monomeric proteins. Minor amounts of dimeric suc1 proteins were also found, but no equilibrium between the two forms was observed and surprisingly, the hexameric assemblies observed in the crystal structure of the human ckshs2 homolog were not detected. These apparent discrepancies between proteins that display cross-complementation address the question of the control of the cks oligomerization process and its link to the biological function.

Key words: Cell cycle regulation; cdc2 kinase subunit, cks protein; Oligomerization; X-ray solution scattering; *Schizosaccharomyces pombe*; *Physarum polycephalum*

1. Introduction

In eukaryotes, cell cycle regulation is a complex process that involves a growing number of protein partners. The key regulators belong to a family of serine/threonine kinases, the cyclin-dependant kinases (cdk) that control the major cell cycle transitions [1]. The cdk are known to be active in complexes with cyclins, proteins which have the property of periodic cell cycle-regulated synthesis and destruction [2]. The entry and exit from mitosis depend on the activation/deactivation of a heterodimeric complex that contains the cdc2 protein kinase and a B-type cyclin. This complex is regulated in different ways: phosphorylation and dephosphorylation events on the conserved Thr^{14}, Tyr^{15} and Thr^{161} residues of the protein kinase (human cdc2 numbering), the level of the cyclin B protein, which peaks just prior to mitosis and disappears at the end of anaphase, and the interaction with other regulatory proteins [3,4]. In addition to the identified phosphatases, kinases, and cdk inhibitors involved in the regulation of the kinase activity [3,5], other known cdk partners still have unclear functions. Among them are the so-called cks proteins, identified and cloned in several eukaryotic cells, from yeast to human [6–8]. The first member of this family, the product of the *suc1* gene in *Schizosaccharomyces pombe*, was isolated as an extragenic suppressor of certain cdc2 mutations [9]. Human cells contain two cks isoforms, namely ckshs1 and ckshs2, which display different patterns of mRNA accumulation through the cell cycle [7]. The ubiquity and the ability of the cks proteins to bind strongly to the cdc2 mitotic kinase, and to the cdk2 and cdk3-related kinases [10], reinforced the idea that these proteins could play an important role in the cell cycle regulation.

In both *S. pombe* and *Saccharomyces cerevisiae*, the elimination of *cks* gene function by construction of null mutation, demonstrated that *suc1* and *cks1* are essential genes [11–13]. Furthermore, overexpression of the cks proteins delays cell division by preventing the dephosphorylation of Tyr^{15} on cdc2, which is a necessary event for the kinase activation [14,15]. However, in vitro, the addition of cks proteins to *Xenopus* extracts containing active cdc2 does not inhibit the kinase activity [14], suggesting that the cks proteins have no effect on activated kinase but on the kinase activation process. These genetic and biochemical results emphasize the inhibitory role of cks proteins on cdk activation but also, paradoxically, their necessity for the proper progress of mitosis.

The cks proteins are conserved at the primary structure level, showing 50–80% sequence identity to one another. Their sizes vary significantly, from 79 to 150 residues, as a consequence of sequences inserted outside the conserved regions (Fig. 1). The location of the cks binding sites on the human cdc2 kinase have been partially identified using alanine scanning mutagenesis [16,17]. Nevertheless the molecular basis of the interaction between the cks proteins and the kinases is still not clear and qualitative and quantitative data concerning the cks proteins and the cdc2/cks complex are missing.

New insights into the mechanism of cks function arose from the X-ray structure determination of the human ckshs2 protein (p9^{ckshs2}) [18]. Crystals of ckshs2 contained hexameric assemblies in which dimers of cks subunits were formed through the exchange β strands containing the conserved HXPEPHILLFRX sequence (residues 60→71). This unexpected hexameric form led to the new hypothesis that cks could act as a hub for the cdk's oligomerization to potentiate the kinase activity in

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Abbreviations: cks, cdc2 kinase subunit; SAXS, small-angle X-ray scattering; cdk, cyclin-dependant kinase; R_s, Stokes radius; R_g, radius of gyration; GA, glutaraldehyde.

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vivo. In that work, it was also reported that ckshs2 existed as an interconverting mixture of monomers, dimers and hexamers in well-defined solution conditions.

As both the human and the fission yeast cks homologs are able to complement a deletion of the *cks1* gene in *S. cerevisiae*, it could be assumed that the ckshs2 hexamerization process should be shared by all the members of the cks family. The recently cloned cksphy protein ($p9^{cksphy}$) from the myxomycete *Physarum polycephalum* (Birck et al., unpublished), that was found 80% identical to $p9^{ckshs2}$ at the primary structure level, and the suc1 protein ($p13^{suc1}$) from *S. pombe*, are representative of the size disparity within this protein family (Fig. 1). Both proteins were expected to mimic the oligomerization state of their human counterpart and their molecular behavior in solution was investigated using small-angle X-ray scattering coupled with biochemical methods.

2. Materials and methods

2.1. Protein purification

The plasmid pRK172, containing the fission yeast *suc1* gene was kindly provided by B. Ducommun (CNRS, Toulouse). $p13^{suc1}$ was bacterially expressed and purified as described by Brizuela et al. [15], with slight modifications. A description of the cksphy cDNA cloning and recombinant DNA manipulations will be published elsewhere. Briefly, cksphy cDNA was subcloned into a T7 promotor-based expression vector. $p9^{cksphy}$ and $p13^{suc1}$ were overexpressed in *Escherichia coli* strain BL21(DE3)pLysS [19]. After induction by addition of 0.4 mM IPTG, bacteria were harvested, resuspended in 1% v/v (relative to the culture volume) of lysis buffer (50 mM Tris-HCl, pH 8, 5 mM EDTA, 10% glycerol, 0.1 mM PMSF, 10 μg/ml of TLCK, TPCK, aprotinin, pepstatin and leupeptin, 0.15% Triton X-100), and sonicated. The crude extract was then centrifuged at 120,000 × *g* for 30 min, and the insoluble material was discarded.

For $p9^{cksphy}$ purification, nucleic acids were precipitated by addition of 18% v/v of a 50 mg/ml protamine sulfate solution pH 7 to the soluble fraction. After stirring, sample was centrifuged for 10 min at 12,000 × *g*, 4°C, and the pellet discarded. The supernatant was then filtered on a 0.22 μm Mylex filter and loaded on a 2.6 × 100 cm Sephacryl S-100 HR column, equilibrated with 50 mM Tris-HCl, pH 8, 1 mM EDTA. The column was eluted at a flow rate of 0.3 ml/min and fractions of 4.5 ml were collected and analysed on 8–25% gradient SDS-polyacrylamide gels. The fractions containing $p9^{cksphy}$ were pooled, dialyzed twice against 50 mM Tris-HCl, pH 9, 1 mM EDTA (Q buffer) and loaded on a Q-sepharose column (15 ml of resin) equilibrated with Q buffer. After washing with 10 ml of Q buffer, a linear gradient of 0–500 mM NaCl in Q buffer was applied. Fractions of 1 ml were collected. The $p9^{cksphy}$ fractions eluted at 240 mM NaCl. They were pooled and dialyzed against 50 mM MES, pH 6.5, 1 mM EDTA (S buffer), before loading on a 5 × 50 mm Mono-S column (Pharmacia). The protein was eluted with a linear gradient of 0–500 mM NaCl in S buffer.

For $p13^{suc1}$ purification, the first two columns were sufficient to obtain pure protein. In this case, the Q buffer was at pH 8.5 (Q_1 buffer), and the protein eluted at 0.32 M NaCl with a linear gradient of NaCl 0 to 1 M in Q_1 buffer.

2.2. FPLC-size-exclusion chromatography

Analytical size-exclusion chromatography measurements were performed at 4°C on a Superdex 75 10/30 column (Pharmacia). The column was equilibrated with 50 mM Tris-HCl, pH 7.2, 150 mM NaCl. Elution flow rate was 0.5 ml/min. (0.5 ml fractions). Volumes of 500 μl were injected with protein concentrations ranging from 0.1 to 5 mg/ml. The column was calibrated using bovine serum albumin (M_r = 67 kDa, R_s = 35.5 Å), ovalbumin (M_r = 43 kDa, R_s = 30.5 Å), chymotrypsinogen B (M_r = 25 kDa, R_s = 20.9 Å), ribonuclease A (M_r = 13.7 kDa, R_s = 16.4 Å) from the Pharmacia low molecular weight calibration kit and aprotinin (6.5 kDa). The $p9^{cksphy}$ and $p13^{suc1}$ Stokes radii (R_s) were determined from the plot of $(-\log Kav)^{1/2}$ versus R_s. The elution volumes were 13.7 ml for $p9^{cksphy}$, and 10.5 ml and 12.1 ml respectively for the two $p13^{suc1}$ species.

2.3. Solution X-ray scattering

Stock protein solutions (6 mg/ml) were stored in 20 mM Tris-HCl, pH 7.2. For SAXS measurements, the proteins were added to the media listed in Table 1. The protein concentration in each experiment was determined spectrophotometrically ($\varepsilon_{1\%/cm}$ = 18.6 for $p9^{cksphy}$, $\varepsilon_{1\%/cm}$ = 15.2 for $p13^{suc1}$).

X-ray scattering curves were recorded on the small angle scattering instrument D24 using synchrotron radiation at LURE-DCI, Orsay. The instrument [20] and the data acquisition system [21] have already been described. The wavelength of the X-rays was 1.488 Å (K-edge of Ni). Scattered intensities were recorded in the angular range 0.0025 $Å^{-1} < s < 0.04$ $Å^{-1}$. All samples contained 1 mM DTT to eliminate the free radicals formed in solution under X-ray irradiation. Four or eight successive frames of 200 s each were recorded for each sample. Each frame was visually inspected and no difference could be detected between successive frames, showing that neither the global structure nor the association state of the protein was altered during data acquisition. The scattering intensity of a reference sample of carbon black recorded immediately before and after each sample was used to normalize all data to the transmitted intensity. The average and standard deviation were then computed for each sample and the scattering from buffer was subtracted before further analysis.

The radius of gyration (R_g) was deduced from Guinier's law:

$$I(s) = I(0) \exp(-4\pi^2 R_g{}^2 s^2/3)$$

where $I(s)$ is the SAXS intensity, $I(0)$ is the value of the extrapolated scattered intensity at s = 0, and the scattering vector $s = (2\sin\theta)/\lambda$, 2θ and λ are the scattering angle and wavelength of the X-rays [22].

For monodisperse solutions, the Guinier plot of ln $I(s)$ versus s^2 should approximate a straight line with a slope of R_g in the small-angle region. After normalization to protein concentration, the value of $I(0)/c$ is proportional to the molecular weight of the scattering entity. Those values are then calibrated using a monomeric lysozyme solution of known concentration as a reference. Measurements with lysozyme were performed using identical experimental settings as those used for $p9^{cksphy}$ and $p13^{suc1}$.

2.4. Cross-linking experiments

Purified proteins (2 μg) were incubated with glutaraldehyde for 30 min at 30°C in 30 μl final volume adjusted with buffer (50 mM Tris-HCl, pH 7.5). Glutaraldehyde (8% in water) was purchased from Fluka. Proteins were cross-linked with 0 to 0.2% freshly diluted glutaraldehyde (GA%). The reaction products were analyzed on 8 to 25% SDS-polyacrylamide gels [23]. The proteins were revealed with colloidal Coomassie blue R250 or silver staining [24].

3. Results

3.1. Analytical gel filtration measurements

After purification, our recombinant $p9^{cksphy}$ and $p13^{suc1}$ proteins were at least 99% pure as judged by SDS-PAGE (Fig. 2A). Using the Superdex 75 column, purified $p9^{cksphy}$ eluted between the 13.7 kDa and 6.5 kDa calibration proteins (Fig. 2B) but purified $p13^{suc1}$ eluted in two peaks between 43 kDa and 13.7 kDa (Fig. 2C). The elution volumes of $p9^{cksphy}$ and the two $p13^{suc1}$ species were used to calculate their hydrodynamic radii. The R_s values were 14.4 Å for $p9^{cksphy}$, and 19.2 Å. and 26.4 Å for the two $p13^{suc1}$ species. These R_s values are in the expected order of magnitude for monomeric $p9^{cksphy}$ and $p13^{suc1}$ and for dimeric $p13^{suc1}$.

3.2. Characterization of cks oligomeric forms by cross-linking

Chemical cross-linking experiments were carried out to characterize the oligomeric state of the two $p13^{suc1}$ peak fractions separated by gel filtration. Fractions containing either the high or the low molecular weight forms of $p13^{suc1}$ were cross-linked using up to 0.2% GA and the products were resolved by SDS-PAGE. We found that the cross-linked high molecular weight form of $p13^{suc1}$ migrated as a 26 kDa band (Fig. 3, lane 8)

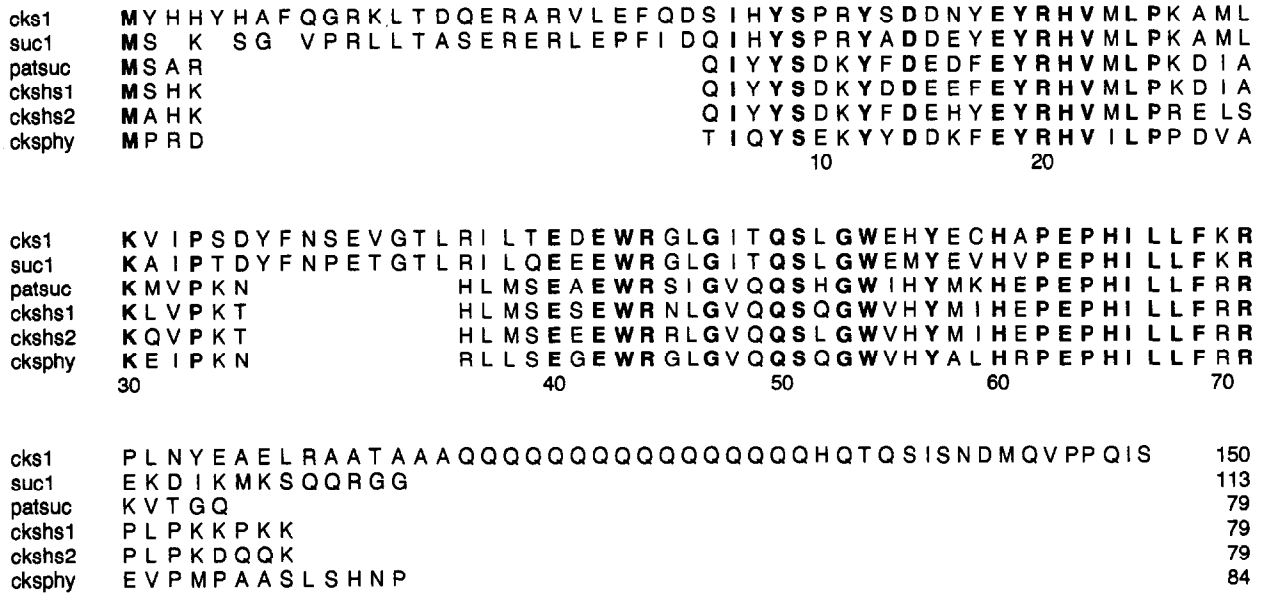


Fig. 1. Alignment of the *Physarum* cksphy amino acid sequence with the known cks homologs: *S. cerevisiae* cks1 [6]; *S. pombe* suc1 [12]; *Patella vulgata* patsuc [8]; human ckshs1 and ckshs2 [7]. Boldface residues are the consensus residues derived from the alignment and the numbering of amino acids refers to the human sequences.

corresponding to protein dimers. In support of this, we found that the low molecular weight form of $p13^{suc1}$ did not form the 26 kDa product when treated with GA under identical conditions (Fig. 3, lane 4).

3.3. SAXS determination of the radius of gyration and molecular weight

Different protein concentrations and several media were used in the SAXS experiments in order to observe the tendency of the monomeric protein to form oligomers as a function of pH or salts (Table 1). In all cases, the protein solutions were found monodispersed and the scattered intensities showed no significant deviation from linearity in the Guinier plots, even at the smallest scattering angles (Fig. 4).

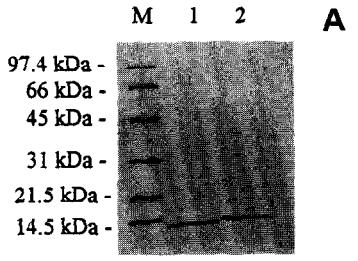
In all conditions, Guinier plots for $p9^{cksphy}$ gave a similar radius of gyration. The average value is 15.8 ± 0.4 Å. Similar conclusions were reached with $p13^{suc1}$ for which the R_g value is 16.8 ± 0.4 Å. The values for these monomeric species are slightly higher than the radius of gyration of the globular and monomeric lysozyme molecule (M_r = 14 305 Da, R_g = 14.2 Å). The extrapolated *I(0)* were brought to absolute scale by measuring small-angle X-ray scattering of a lysozyme solution of known concentration. It allowed us to calculate the molecular weight of the monomeric $p9^{cksphy}$ and $p13^{suc1}$ proteins, 12,410 Da ± 1,200 and 13,355 Da ± 1,100, respectively. Compared to the molecular weight of the proteins deduced from cDNA sequencing (9,936 Da and 13,447 Da for $p9^{cksphy}$ and $p13^{suc1}$, respectively), the value for $p9^{cksphy}$ is slightly higher than expected. Nevertheless, this cannot be related to the presence of oligomeric species that would have been observed by the other methods.

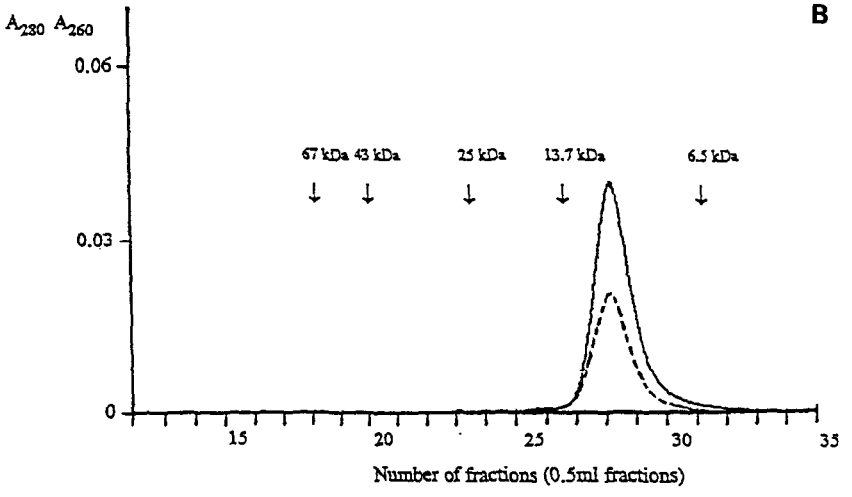
4. Discussion

The purification scheme developed for $p9^{cksphy}$ and $p13^{suc1}$ yielded highly pure proteins as judged by SDS-PAGE analysis (Fig. 2A). Analytical size-exclusion chromatography performed on purified $p13^{suc1}$ revealed that, in the native state, this protein exists as both monomers and dimers. With $p9^{cksphy}$, in the same protein concentration range, we could only detect monomeric protein. From the cross-linking experiments, the monomeric and dimeric character of $p13^{suc1}$ seemed unambiguous (Fig. 3).

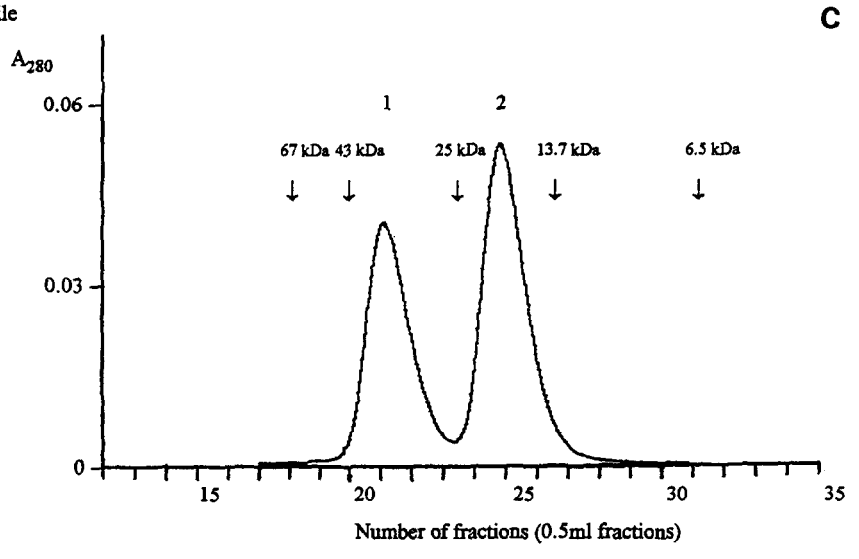
The methods used in this work to characterize the oligomerization states of the two cks proteins gave consistent results. SAXS was chosen because it is a non-destructive method to describe the macromolecular organization of protein solutions and its conjunction with more classical biochemical tools, gave identical conclusions for protein concentrations ranging from 0.1 to 6 mg/ml. $p9^{cksphy}$ and the major pool of $p13^{suc1}$ exist as monomeric proteins in solution. This state is independent of pH from 5.5 to 8.5, ionic strength up to 0.5 M and of time. In all experiments, the Guinier plots gave R_g values that fitted to monomeric proteins and that are in the order of magnitude of other proteins of similar molecular weight (e.g. crotapotin, M_r = 9 kDa, R_g = 13.6 Å [25]; lysozyme, M_r = 14.3 kDa, R_g = 14.2 Å (this work); calmodulin, M_r = 17 kDa, R_g = 21.4 Å [26]). The molecular weight determination from SAXS was in good agreement with the expected value, reinforcing the idea that the linearity of the Guinier plots corresponded to true monodispersed solutions at the protein concentrations used for the measurements (2 to 4.5 mg/ml). These concentrations were chosen such that the signal to noise ratio for X-ray scattering would be good while avoiding non-specific aggregation that may occur at high protein concentrations. In any case, it can be assumed that a concentration of 3 mg/ml (0.3 mM) is significantly higher than the cellular concentration of the protein, which was estimated to be in the micromolar range [13], and no accumulation of the protein at a specific location in the cell was clearly established [27–29].

The R_g values for monomeric $p9^{cksphy}$ (15.8 Å) and $p13^{suc1}$ (16.8 Å) are above those measured for crotapotin and lysozyme. The molecular envelope of the crotapotin was described as an oblate ellipsoid of revolution with semi-axes





1. Elution profile



2. SDS-PAGE analysis

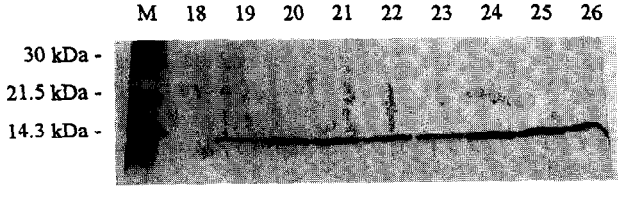


Fig. 2. (A) SDS-PAGE (8–20%), stained with Coomassie blue, showing recombinant $p9^{cksphy}$ (lane 1) and $p13^{sucl}$ (lane 2) after purification. Markers are shown on the left. (B) Elution profile of FPLC size-exclusion chromatography with pure $p9^{cksphy}$ protein. The protein eluted in a single peak as monomeric species. Arrows indicate the elution positions of the protein calibration markers. The absorbance (A) was monitored at 280 nm (bold line) and 260 nm (dashed line). The detailed chromatographic conditions are described in materials and methods. (C) FPLC size-exclusion chromatography analysis of $p13^{sucl}$ proteins. 1. $p13^{sucl}$ eluted in two peaks at apparent molecular weights corresponding to potential dimers (peak 1) and monomers (peak 2). Arrows indicate the elution positions of the protein calibration markers. The detailed chromatographic conditions are described in materials and methods. 2. Fractions (20 μl aliquot) were analysed by gel electrophoresis and silver staining. Markers (M) are shown on the left. ←

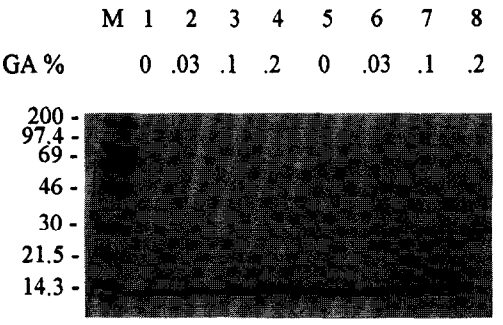


Fig. 3. Cross-linking experiments with high and low molecular weight $p13^{sucl}$ fractions. Reactions were performed on fractions from gel filtration (Fig. 2C), fraction 24 (lane 1–4) and fraction 20 (lane 5–8), and analyzed by SDS-PAGE.

$a = b = 22$ Å and $c = 10$ Å [25] and those of lysozyme as a prolate ellipsoid with $a = 45$ Å and $b = c = 30$ Å [30]. For $p13^{sucl}$ and $p9^{cksphy}$, we can reject the simplest spherical model hypothesis because, assuming 20% hydration, this shape would imply that the Stokes radius is 1.290 R_g. The values therefore computed from the experimental R_g of monomeric forms, are significantly different (19.9 Å and 21.2 Å for $p9^{cksphy}$ and $p13^{sucl}$, respectively) from those deduced from analytical size-exclusion chromatography (14.4 Å and 19.2 Å). These low experimental R_s values could be explained if the monomeric cks proteins have extended or asymetrical shapes, because it is known that in such cases retarded elution from gel filtration is common [31]. Both methods would thus suggest that the molecular envelope of these cks monomers deviates from a globular shape.

From the X-ray structure, Parge et al. [18] proposed that the protonation of His^{60}, followed by salt bridge formation with Glu^{63} from the second subunit, would promote the dimeric assembly of the ckshs2 protein and explain the equilibrium between monomers, dimers and hexamers. SAXS measurements on $p9^{cksphy}$ and $p13^{sucl}$ made at different pH values around the expected pK_a of the histidine residue gave no evidence for such a pH dependency of dimerization, although these residues are strictly conserved in $p13^{sucl}$ and $p9^{cksphy}$.

The importance of divalent cations for oligomerization was also suggested. Upon dimer formation, the β strands exchange brings the adjacent pairs of Glu^{63} side chains in vicinity, providing a metal binding site and thus a basis for metal-initiated conformational change. Using monomeric proteins, addition of 10 mM $CaCl_2$ or $MgCl_2$ to the medium, (more than 1 mM $ZnCl_2$ led to protein irreversible precipitation), did not lead to the formation of dimers or hexamers according to size-exclusion chromatography analysis. Finally, imidazole-malate buffer at pH 6.6, containing 150 mM NaCl and 10 mM calcium acetate, that was shown to favor ckshs2 hexamer formation in solution, did not induce the formation of higher oligomeric species with $p9^{cksphy}$ and $p13^{sucl}$, using either monomers or dimers in the latter case. This dimer was also found stable with time in several

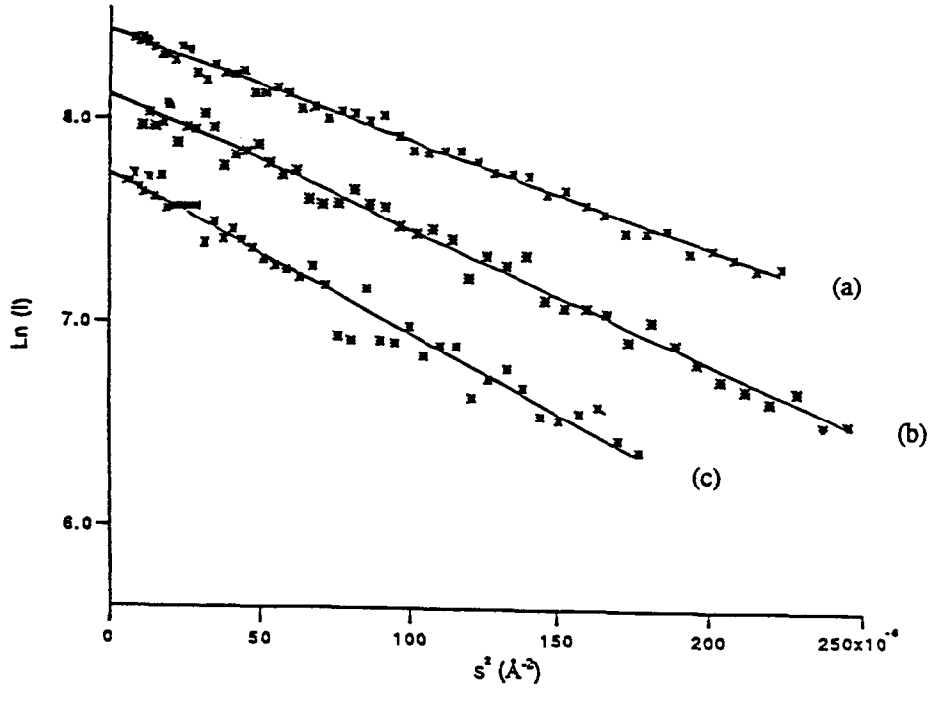


Fig. 4. Guinier plots of small-angle X-ray scattering data extrapolated to zero concentration. Symbols: (a) lysozyme at 4.9 mg/ml in 50 mM Na acetate pH 4.6; (b) $p9^{cksphy}$ at 1.7 mg/ml in 100 mM Tris-HCl, pH 7.2; (c) $p13^{sucl}$ monomeric form at 3.3 mg/ml in 100 mM Tris-HCl, pH 7.2, 100 mM ammonium sulfate. $I(0)$ values for each plot have been displaced for the sake of clarity.

Table 1
Conditions used for $p9^{cksphy}$ (A) and $p13^{sucl}$ (B) SAXS experiments and Rg values respectively measured

A

Buffer	pH	Salt	Additives	R_g (Å)
100 mM Tris-HCl	7.2	–	–	15.7 ± 0.4
50 mM Imidazole-Malate	6.6	0.15 M NaCl	10 mM Ca acetate	16.1 ± 0.25
100 mM MES-NaOH	6.5	0.5 M NaCl	–	15.9 ± 0.8

B

Buffer	pH	Salt	Additives	R_g (Å)
100 mM Tris-HCl	7.2	–	–	16.8 ± 0.2
100 mM Tris-HCl	7.2	0.1 M	–	16.4 ± 0.8
100 mM Tris-HCl	7.2	0.5 M NaSCN	–	17.1 ± 0.8
50 mM immidazole-Malate	6.6	0.15 M NaCl	10 mM Ca acetate	17.5 ± 0.1
100 mM MES-NaOH	6.5	0.5 M NaCl	–	16.5 ± 0.8
100 mM MES-NaOH	6	0.5 M NaCl	–	16.6 ± 0.5
100 mM MES-NaOH	5.5	0.5 M NaCl	–	16.7 ± 0.6

media and there was no evidence for dissociation into monomers nor formation of higher oligomeric species according to size-exclusion chromatography analysis.

The X-ray structure determination of one the human homologs seemed to provide the archetype of the structural organization and a new hypothetical role for the cks family. The cks cross-species complementation, the functionality of the recombinant $p9^{cksphy}$ demonstrated in vivo (C. Birck, unpublished), and the great sequence identity within this protein family would suggest a common structure-function relationship. It was therefore unexpected that the oligomerization states of $p13^{sucl}$ and $p9^{cksphy}$ differed from those observed for the human $p9^{ckshs2}$, all three beeing *E. coli* recombinant proteins. The identical behaviour of $p13^{sucl}$ and $p9^{cksphy}$ monomeric forms suggest that their sequence differences play no role in the stability of the polypeptide chain folding and the observation of a dimeric form only in the case of $p13^{sucl}$ might be related to the lower level of expression of $p9^{cksphy}$ (5 mg per liter of cell culture) compared to $p13^{sucl}$ (50 mg per liter of cell culture).

During evolution, genes for cdc2-related kinases and cyclins have multiplied and increased the level of complexity of the cell cycle regulation. The presence of two cks isoforms is so far specific to human cells and the hexameric assembly observed with one of the two human proteins could be part of the specificity of higher organisms. The stability of the monomeric and dimeric forms characterized in this work raises the questions of which events eventually trigger the oligomerization process and what is the nature of the cks-cdc2 complexes. Work is in progress to characterize better the relationship between the oligomerization state and the biophysical and biological properties in the cks protein family.

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